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METHODS, COMPOSITIONS AND GENETIC SEQUENCES FOR MODULATING FLOWERING IN PLANTS, AND PLANTS GENETICALLY ~~MODIFIED TO FLOWER EARLY AND TARDILY~~

BACKGROUND OF THE INVENTION

a) Field of the invention

The invention relates to methods, compositions and genetic sequences to modulate flowering in plants and to plants genetically modified to flower early and plants genetically modified to flower tardily.

b) Brief description of the prior art

In plants, the transition from vegetative to reproductive growth involves complex interactions between several endogenous biochemical pathways. These pathways are continuously evaluating the environmental conditions and the state of growth of the plant. When adequate conditions are met, cross-talk between pathways will ultimately result in the formation of a floral meristem.

For a long time, plant scientists have tried to control floral induction. The results of grafting experiments performed about 70 years ago led to the proposal of a hypothesis which states that a flower inducer, i.e. "a florigen", is synthesized in the leaves and translocated to the shoot apex to induce the development of the flower meristem. However, despite considerable research efforts, the search for the hypothetical "florigen" hormone was unsuccessful. Molecules such as cytokinins, gibberellins and carbon assimilates have also been proposed to act as flowering promoters in some species. For instance, U.S. patents Nos. 5,523,281; 6,020,288 and 6,057,157 disclose various methods and compositions for inducing, accelerating and prolonging flowering in plants or enhancing their growth. However, some of the molecules or compositions described in these patents were found to be inactive or even inhibitory to flower formation in other plant species. Some others are also known to affect the biomass or the plant morphology.

12-hydroxyjasmonic acid (see Fig. 1A) is a natural metabolite and was first isolated from the leaves of *Solanum tuberosum* (potato) (Yoshihara *et al.* (1989),

Agric. Biol. Chem. 53: 2835). The biosynthesis of 12-hydroxyjasmonic acid has not been studied at the biochemical level but recent studies suggest that jasmonic acid is converted to 12-hydroxyjasmonic acid by a single oxidation step catalyzed by the jasmonic acid 12-hydroxylase (Yoshihara *et al.* (1996), Plant Cell Physiol. 37: 586). 11-hydroxyjasmonic acid (see Fig. 1B) is also a natural metabolite for which the mechanism of biosynthesis have not been described either. However, based on the results obtained for the *in vivo* synthesis of 12-hydroxyjasmonic acid, one can predict that a jasmonic acid 11-hydroxylase converts jasmonic acid or methyljasmonic acid into the 11-hydroxylated compounds.

Although many functions have been associated with jasmonates metabolites such as 12-hydroxyjasmonic acid and/or 11-hydroxyjasmonic acid, these metabolites have never been associated with flower formation. For instance, U.S. patent No 5,935,809, suggests the use of jasmonate for inducing plant defense mechanisms. U.S. patent No 5,814,581 describes a plant growth promoter composition comprising jasmonate and brassinolide as active ingredients and Japanese patent application No 00292220 (A) published April 3, 1990, Yoshihara *et al.* (1989), *Agric. Biol. Chem.* 53: 2835-2837, Matsuki *et al.* (1992), Biosci. Biotech. Biochem. 56: 1329.; and Koda and Okazawa (1988), *Plant Cell Physiol.* 29: 969), suggest the use of 12-hydroxyjasmonic acid for inducing tuber formation in potatoes. None of these documents disclose nor suggest that compounds of the jasmonates family are involved in flower formation pathways.

Accordingly, there is a need for effective methods and compositions to modulate flowering, particularly for plants which are used in the food-processing industry and plants with a horticultural value. There is also a need for plants genetically modified to flower early and for plants genetically modified to flower tardily as well as for methods for producing such genetically modified plants.

SUMMARY OF THE INVENTION

The present invention relates to the modulation of flowering in plants. More particularly, the present invention pertains to methods, compositions and genetic sequences for modulating flowering in plants and to plants genetically modified to flower early and to plants genetically modified to flower tardily.

According to an aspect of the invention, there is provided a method for modulating flowering in a plant. The method comprises the step of modifying in said plant the endogenous level of at least one compound of the jasmonate family, and more particularly compounds selected from the group consisting of jasmonic acid, jasmonic acid-tyrosine conjugate, jasmonic acid-tryptophan conjugate, jasmonic acid-phenylalanine conjugate, jasmonic acid-isoleucine conjugate, jasmonic acid-leucine conjugate, jasmonic acid-valine conjugate, 12-hydroxyjasmonic acid, glucoside of 12-hydroxyjasmonic acid, sulfate ester of 12-hydroxyjasmonic acid, methyljasmonic acid, 12-hydroxymethyljasmonic acid, glucoside of 12-hydroxymethyljasmonic acid, sulfate ester of 12-hydroxymethyljasmonic acid, 11-hydroxyjasmonic acid, glucoside of 11-hydroxyjasmonic acid, sulfate ester of 11-hydroxyjasmonic acid, 11-hydroxymethyljasmonic acid, glucoside of 11-hydroxymethyljasmonic acid, sulfate ester of 11-hydroxymethyljasmonic acid, and mixtures thereof.

According to another aspect of the invention, flowering of a plant is induced by increasing in the plant the endogenous level of at one flowering inducing compound selected from the previously mentioned jasmonate family compounds excluding sulfate ester of 12-hydroxyjasmonic acid, sulfate ester of 12-hydroxymethyljasmonic acid, sulfate ester of 11-hydroxyjasmonic acid, and sulfate ester of 11-hydroxymethyljasmonic acid. In a preferred embodiment, this can be achieved by:

a) applying to the plant at least one flowering inducing compound and/or salts thereof;
b) applying to the plant at least one inhibitor of a sulfotransferase sulfonating 12-hydroxyjasmonic acid and/or 11-hydroxyjasmonic acid;
c) applying to the plant at least one stimulator of an hydroxylase hydroxylating jasmonic acid and/or methyljasmonic acid;
d) increasing in the plant the endogenous level of an hydroxylase hydroxylating jasmonic acid and/or methyljasmonic acid; and/or
e) lowering in the plant the endogenous level of a sulfotransferase sulfonating 12-hydroxyjasmonic acid and/or 11-hydroxyjasmonic acid.

Alternatively, flowering of a plant can be delayed by lowering the endogenous level in the plant of at least one of the above mentioned flowering inducing compounds. According to an embodiment of the invention this can be achieved by:

a) applying to the plant an inhibitor and/or an inactivator of at least one of the flowering inducing compounds;
b) applying to the plant at least one stimulator of a sulfotransferase sulfonating 12-hydroxyjasmonic acid and/or 11-hydroxyjasmonic;
c) applying to the plant at least one inhibitor of an hydroxylase hydroxylating jasmonic acid and/or methyljasmonic acid;
d) lowering in the plant the endogenous level of an hydroxylase hydroxylating jasmonic acid and/or methyljasmonic acid; and/or
e) increasing in the plant the endogenous level of a sulfotransferase sulfonating 12-hydroxyjasmonic acid and/or 11-hydroxyjasmonic acid.

According to another aspect of the invention, compositions for modulating flowering are provided. In an embodiment of the invention, a composition for inducing flowering in a plant is provided, comprising a flowering inducing effective amount of at least one of the previously mentioned flowering inducing compounds or salts thereof, in combination with a diluent or a carrier such that an induction in flowering of the plant occurs when compared to a corresponding plant in the absence of the flowering inducing composition. Similarly, in another embodiment, is provided a flowering delaying composition for delaying flowering in a plant, the composition comprising a flowering delaying effective amount of an inhibitor or of an inactivator of the previously mentioned jasmonate family compounds excluding sulfate ester of 12-hydroxyjasmonic acid, sulfate ester of 12-hydroxymethyljasmonic acid, sulfate ester of 11-hydroxyjasmonic acid, and sulfate ester of 11-hydroxymethyljasmonic acid, in combination with a diluent or a carrier such that a delay in flowering of said plant occurs when compared to a corresponding plant in the absence of the flowering delaying composition.

According to a further aspect of the invention, there are provided genetically modified plants. In an embodiment, a plant is genetically modified to flower early when compared to a corresponding plant not genetically modified. The genetically

modified plant exhibits an increased endogenous level of at least one compound selected from the previously mentioned jasmonate family compounds, excluding sulfate ester of 12-hydroxyjasmonic acid, sulfate ester of 12-hydroxymethyljasmonic acid, sulfate ester of 11-hydroxyjasmonic acid, and sulfate ester of 11-hydroxymethyljasmonic acid, when compared to a corresponding non-genetically modified plant. In another embodiment a plant is genetically modified to flower tardily when compared to a corresponding plant not genetically modified, the genetically modified plant exhibiting a lowered level of at least one compound selected from the previously mentioned jasmonate family compounds excluding sulfate ester of 12-hydroxyjasmonic acid, sulfate ester of 12-hydroxymethyljasmonic acid, sulfate ester of 11-hydroxyjasmonic acid, and sulfate ester of 11-hydroxymethyljasmonic acid.

In another aspect, the present invention is directed to an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a plant hydroxyjasmonic acid sulfotransferase, and more particularly a plant 11- or 12-hydroxyjasmonic acid sulfotransferase. Preferably, the nucleotide sequence is selected from the group consisting of SEQ ID NO:1, nucleotide sequences having at least 50% similarity with SEQ ID NO:1, SEQ ID NO:2 nucleotide sequences having at least 50% similarity with SEQ ID NO:2, and sequences hybridizing under low stringency conditions to one or more of these sequences. Advantageously, these sequences are incorporated into a vector.

According to a related aspect, the invention provides transgenic plants incorporating at least one of these nucleotide sequences so that the transgenic plants are capable of flowering early or tardily. The invention also provides methods for producing such transgenic plants.

An advantage of the present invention is that it allows to modulate flowering in plants without decreasing yield or modifying plant morphology. According to the invention it is possible to inhibit flowering in crop plants such as sugarcane, sugar beets or lettuce, just to mention a few, and thereby increase the taste, sweetness, and tenderness of these agricultural products. On the other hand, it is also possible according to the present invention, to induce flowering which is an

advantage of great economic importance for horticultural plants and some crop plants such as cauliflower and broccoli.

Other objects and advantages of the present invention will be apparent upon reading the following non-restrictive description of several preferred embodiments, made with reference to the accompanying drawings and to the enclosed examples.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and **1B** show the chemical structures of 12-hydroxyjasmonic acid (Fig. 1A) and 11-hydroxyjasmonic acid (Fig. 1B).

Figures 2A and **2B** are pictures showing the effect on flowering time of a treatment with 12-hydroxyjasmonic acid (Fig. 2B) in *Arabidopsis thaliana*, when compared to a treatment with water (Fig. 2A).

Figure 3 is a picture showing the phenotype of transgenic *Arabidopsis* plants expressing *AtST2a* gene under the control of a constitutive promoter when compared to wild type non-transgenic plant (WT). S5, S6, S9, and S16 indicate independent transgenic lines.

Figure 4 is a Western blot of protein extracts from the plants shown in Fig. 3 probed with anti-*AtST2a* antibodies. MW: Molecular weight markers; WT: wild type plants; S5, S6, S9, and S16: independent transgenic lines.

Figure 5 is a picture showing the phenotype of transgenic *Arabidopsis* plants expressing the *AtST2a* gene in the antisense orientation under the control of a constitutive promoter (TL 7-2-5) when compared to non transgenic plants (WT).

Figure 6 is a picture showing the effect of methyljasmonic acid treatment on the flowering time of wild type *Arabidopsis thaliana* plants (WT C24) and on transgenic *Arabidopsis thaliana* plants expressing the *AtST2a* gene in the antisense orientation under the control of a constitutive promoter (TL 7-2-5).

Figure 7: Shows nucleotide sequence of *AtST2a* gene (SEQ ID NO 1) taken from *Arabidopsis thaliana* database at Stanford University (clone number M0J9, gene MOJ9.16 and the EST 119G6T7) and the GenBank™ database (accession number AB010697, nucleotides 53936 to 55015).

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Figure 8: Shows the deduced amino acid sequence (SEQ ID NO 3) of the protein encoded by the *AtST2a* gene shown in Fig. 7.

Figure 9: Shows the nucleotide sequence of *AtST2b* gene (SEQ ID NO 2) taken from *Arabidopsis thaliana* database at Stanford University (clone number M0J9, gene MOJ9.15) and the GenBank™ database (accession number AB010697, nucleotides 50627 to 51670).

Figure 10: Shows the deduced amino acid sequence (SEQ ID NO 4) of the protein encoded by the *AtST2b* gene shown in Fig. 9.

Figure 11 is a Northern blot of plants mRNA extracts showing the effect of various 12-hydroxyjasmonate concentrations on the expression of the *AtST2a* gene.

Figure 12 is a Northern blot of plants mRNA extracts showing the effect of the photoperiod on the expression of the *AtST2a* gene.

DETAILED DESCRIPTION OF THE INVENTION

A) Definitions

In order to provide an even clearer and more consistent understanding of the specification and the claims, including the scope given herein to such terms, the following definitions are provided:

11-hydroxyjasmonic acid: 3-Oxo-2-(4-hydroxy-2-pentenyl)-cyclopentane-1-acetic acid. Its chemical structure is shown in Fig. 1B.

11-hydroxyjasmonic acid glucoside: 3-Oxo-2-(4-β-D-glucopyranosyloxy-2-pentenyl)-cyclopentane-1-acetic acid

11-hydroxyjasmonic acid sulfate: 3-Oxo-2-(4-hydroxysulfonyloxy-2-pentenyl)-cyclopentane-1-acetic acid

12-hydroxyjasmonic acid: 3-Oxo-2-(5-hydroxy-2-pentenyl)-cyclopentane-1-acetic acid. Its chemical structure is shown in Fig. 1A.

12-hydroxyjasmonic acid glucoside: 3-Oxo-2-(5-β-D-glucopyranosyloxy-2-pentenyl)-cyclopentane-1-acetic acid.

12-hydroxyjasmonic acid sulfate: 3-Oxo-2-(5-hydroxysulfonyloxy-2-pentenyl)-cyclopentane-1-acetic acid.

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Antisense: Refers to nucleic acids molecules capable of regulating the expression of a corresponding gene in a plant. An antisense molecule as used herein may also encompass a gene construct comprising a structural genomic gene, a cDNA gene or part thereof in reverse orientation relative to its or another promoter. Typically antisense nucleic acid sequences are not templates for protein synthesis but yet interact with complementary sequences in other molecules (such as a gene or RNA) thereby causing the function of those molecules to be affected.

Delay or **retard** or **tardily**: When used in conjunction with the term flowering, it refers to the increase of the time of vegetative growth before flowering of a plant. A flowering delay may be observed when compared with a corresponding plant where flowering has not been delayed.

Effective amount: Refers to the amount or concentration of a suitable compound that is administered to a plant such that the compound induces or delays flowering of a plant.

Exogenous nucleic acid: A nucleic acid sequence (such as cDNA, cDNA fragments, genomic DNA fragments, antisense RNA, oligonucleotide) which is not normally part of a plant genome. The "exogenous nucleic acid" may be from any organism or purely synthetic. Typically, the "exogenous nucleic acid sequence" encodes a plant gene such as a *AtST2a*, *AtST2b* or functional homologues of these genes.

Expression: The process whereby an exogenous nucleic acid, such as a nucleic acid sequence encoding a gene, is transcribed into a mRNA and afterwards translated into a peptide or a protein, in order to carry out its function, if any.

Flowering: Refers to the appearance of a flower bud in a plant. As it is known, the flower bud will eventually mature to a flower.

Functional homologue: Refers to a molecule having at least 50%, more preferably at least 55%, even more preferably at least 60%, still more preferably at least 65-70%, and yet even more preferably greater than 85% similarity at the level of nucleotide or amino acid sequence to at least one or more regions of a given nucleotide or amino acid sequence. According to preferred embodiments of the present invention, the terms "functional homologue" refer to proteins or nucleic

acid sequences encoding an enzyme having a substantially similar biological activity as 11- or 12-hydroxyjasmonate sulfotransferase and isoenzyme(s) thereof. Such a functional homologue may exist naturally or may be obtained following a single or multiple amino acid substitutions, deletions and/or additions relative to the naturally occurring enzyme(s) using methods and principles well known in the art. A functional homologue of a protein may or may not contain post-translational modifications such as covalently linked carbohydrate, if such modification is not necessary for the performance of a specific function. It should be noted, however, that nucleotide or amino acid sequences may have similarities below the above given percentages and still encode a 11- or 12-hydroxyjasmonate sulfotransferase-like molecule, and such molecules may still be considered within the scope of the present invention where they have regions of sequence conservation.

Genetic/nucleotide sequence: These terms are used herein in their most general sense and encompass any contiguous series of nucleotide bases encoding directly, or via a complementary series of bases, a sequence of amino acids comprising a hydroxyjasmonic acid sulfotransferase molecule, and more particularly a 11- or 12-hydroxyjasmonic acid sulfotransferase. Such a sequence of amino acids may constitute a full-length 11- or 12-hydroxyjasmonic acid sulfotransferase such as is set forth in SEQ ID No:1 and SEQ ID No:2 or an active truncated form thereof or a functional mutant, derivative, part, fragment, homologue or analogue thereof, or may correspond to a particular region such as an N-terminal, C-terminal or internal portion of the enzyme.

Genetic modification or genetic engineering: Refers to the introduction of an exogenous nucleic acid into one or more plant cells to create a genetically modified plant. Methods for genetically modifying a plant are well known in the art. In some cases, in may be preferable that the genetic modification is permanent such that the genetically modified plant may regenerate into whole, sexually competent, viable genetically modified plants. A plant genetically modified in a permanent manner would preferably be capable of self-pollination or cross-pollination with other plants of the same species, so that the exogenous nucleic

acid, carried in the germ line, may be inserted into or bred into agriculturally useful plant varieties.

Endogenous level(s): Refers to the concentration of a given substance which is normally found in a plant (intrinsic) at a given time and stage of growth. Reference herein is made to the altering of the endogenous level of a compound or of an enzyme activity relating to an elevation or reduction in the compound's level or enzyme activity of up to 30% or more preferably of 30-50%, or even more preferably 50-75% or still more preferably 75% or greater above or below the normal endogenous or existing levels. The levels of a compound or the levels of activity of an enzyme can be assayed using known method and techniques.

Isolated nucleic acid molecule: Means a genetic sequence in a non-naturally-occurring condition. Generally, this means isolated away from its natural state or formed by procedures not necessarily encountered in its natural environment. More specifically, it includes nucleic acid molecules formed or maintained *in vitro*, including genomic DNA fragments, recombinant or synthetic molecules and nucleic acids in combination with heterologous nucleic acids such as heterologous nucleic acids fused or operably-linked to the genetic sequences of the present invention. The term "isolated nucleic acid molecule" also extends to the genomic DNA or cDNA or part thereof, encoding a hydroxyjasmonic acid sulfotransferase, preferably a 11- or 12-hydroxyjasmonic acid sulfotransferase, or a functional mutant, derivative, part, fragment, homologue or analogue of 11- or 12-hydroxyjasmonic acid sulfotransferase in reverse orientation relative to its or another promoter. It further extends to naturally-occurring sequences following at least a partial purification relative to other nucleic acid sequences. The term isolated nucleic acid molecule as used herein is understood to have the same meaning as nucleic acid isolate.

Induce or **increase**: When used in conjunction with the term flowering, it refers to the reduction of the time of vegetative growth before flowering of a plant. A flowering induction may be observed when compared with a corresponding plant wherein flowering has not been induced.

Modulation: Refers to the process by which a given variable is regulated to a certain proportion. According to preferred embodiments of the present invention,

the term “modulate” refers in some cases to induction and in other cases delay, of flowering of a plant.

Plant: refers to a whole plant or a part of a plant comprising, for example, a cell of a plant, a tissue of a plant, an explant, or seeds of a plant. This term further contemplates a plant in the form of a suspension culture or a tissue culture including, but not limited to, a culture of calli, protoplasts, embryos, organs, organelles, etc.

Similarity/Complementarity: In the context of nucleic acid sequences, these terms mean a hybridizable similarity under low, alternatively and preferably medium and alternatively and most preferably high stringency conditions, as defined below. Such a nucleic acid is useful, for example, in screening hydroxyjasmonic acid sulfotransferase genetic sequences, preferably a 11- or 12-hydroxyjasmonic acid sulfotransferase genetic sequences from various sources or for monitoring an introduced genetic sequence in a transgenic plant. The preferred oligonucleotide is directed to a conserved hydroxyjasmonic acid sulfotransferase, preferably a 11- or 12-hydroxyjasmonic acid sulfotransferase genetic sequence or a sequence conserved within a plant genus, plant species and/or plant cultivar or variety.

Stringency: For the purpose of defining the level of stringency, reference can conveniently be made to Maniatis et al. (1982) at pages 387-389, and especially paragraph 11. A low stringency is defined herein as being in 4-6X SSC/1% (w/v) SDS at 37-45 °C for 2-3 hours. Depending on the source and concentration of nucleic acid involved in the hybridization, alternative conditions of stringency may be employed such as medium stringent conditions which are considered herein to be 1-4X SSC/0.5-1% (w/v) SDS at greater than or equal to 45°C for 2-3 hours or high stringent conditions considered herein to be 0.1-1X SSC/0.1-1.0% SDS at greater than or equal to 60° C. for 1-3 hours.

Transformed plant: Refers to introduction of an exogenous nucleic acid, typically a gene, into a whole plant or a part thereof, and expression of the exogenous nucleic acid in the plant.

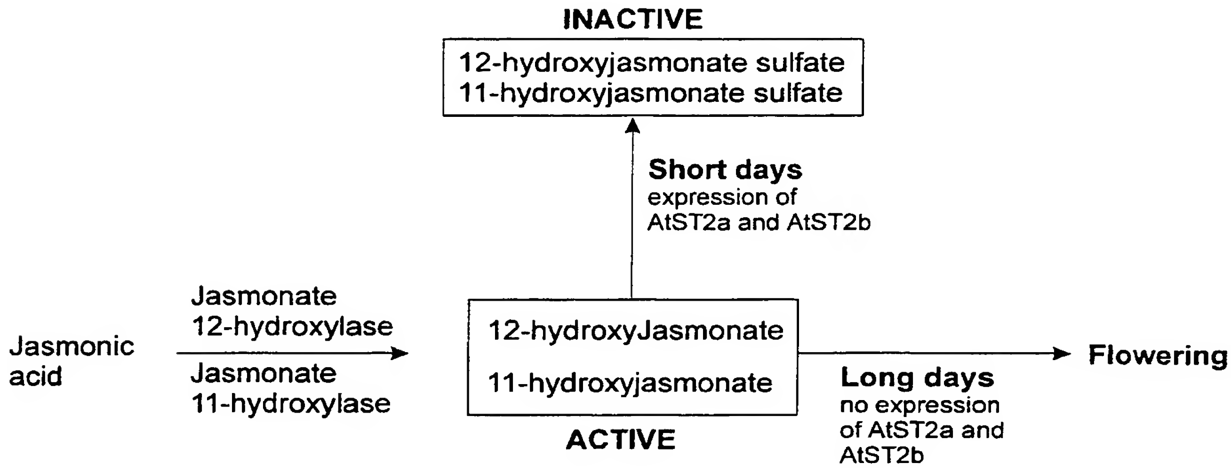
Transgenic plant: Refers to a whole plant or a part thereof stably transformed with an exogenous nucleic acid introduced into the genome of an individual plant cell using genetic engineering methods.

Vector: A self-replicating RNA or DNA molecule which can be used to transfer an RNA or DNA segment from one organism to another. Vectors are particularly useful for manipulating genetic constructs and different vectors may have properties particularly appropriate to express protein(s) in a recipient during cloning procedures and may comprise different selectable markers. Bacterial plasmids are commonly used vectors. Preferably, the vectors of the invention are capable of facilitating transfer of a nucleic acid into a plant cell and/or facilitating integration into a plant genome.

B) General overview of the invention

The present inventors have now discovered that compounds of the jasmonate family are involved in the flowering of plants. They have also characterized the biological function of two highly homologous genes from *A. thaliana* (*AtST2a* and *AtST2b*) which encode enzymes that inactivate by sulfonation the biological activity of 11-hydroxyjasmonic acid and 12-hydroxyjasmonic acid. The inventors have also determine that expression of the *AtST2a* gene in under the control of photoperiod. These properties suggest that flowering could be induced or delayed, and yield to the elaboration of the following model which is given for purposes of clarification and not to limit the scope of the present invention.

Proposed model for the control of flower induction in plants:



Using methods, compositions and genetically modified plants the present application demonstrates that flowering can actually be modulated in plants.

According to an aspect of the invention, flowering of a plant is modulated by modifying in the plant the endogenous level of at least one compound of the jasmonate family selected from the group consisting of jasmonic acid, jasmonic acid-tyrosine conjugate, jasmonic acid-tryptophan conjugate, jasmonic acid-phenylalanine conjugate, jasmonic acid-isoleucine conjugate, jasmonic acid-leucine conjugate, jasmonic acid-valine conjugate, 12-hydroxyjasmonic acid, glucoside of 12-hydroxyjasmonic acid, sulfate ester of 12-hydroxyjasmonic acid, methyljasmonic acid, 12-hydroxymethyljasmonic acid, glucoside of 12-hydroxymethyljasmonic acid, sulfate ester of 12-hydroxymethyljasmonic acid, 11-hydroxyjasmonic acid, glucoside of 11-hydroxyjasmonic acid, sulfate ester of 11-hydroxyjasmonic acid, 11-hydroxymethyljasmonic acid, glucoside of 11-hydroxymethyljasmonic acid, and sulfate ester of 11-hydroxymethyljasmonic acid.

In practice, flowering modulation is achieved in two different ways: it is induced or it is delayed. Although many approaches may be used to achieve these effects, the approaches described hereinafter are preferably used according to the invention.

1) Chemical approach

i) Flowering induction

According to the invention, flowering is induced by increasing in a plant the endogenous level of at least one given flowering inducing compound of the jasmonate family selected preferably from the group consisting of jasmonic acid, jasmonic acid-tyrosine conjugate, jasmonic acid-tryptophan conjugate, jasmonic acid-phenylalanine conjugate, jasmonic acid-isoleucine conjugate, jasmonic acid-leucine conjugate, jasmonic acid-valine conjugate, 12-hydroxyjasmonic acid, glucoside of 12-hydroxyjasmonic acid, methyljasmonic acid, 12-hydroxymethyljasmonic acid, glucoside of 12-hydroxymethyljasmonic acid, 11-hydroxyjasmonic acid, glucoside of 11-hydroxyjasmonic acid, 11-hydroxymethyljasmonic acid, and glucoside of 11-hydroxymethyljasmonic acid. All these compounds have been tested for their biological activity and all of them have been shown to induce flowering at various levels (data not shown). More preferably, levels of 12-hydroxyjasmonic acid and/or 11-hydroxyjasmonic acid are increased. The flowering induction and the endogenous level increased is detectable when compared to a corresponding plant in which the endogenous level of said compound has not been modified. However, it could be preferable in some cases to genetically modify a plant to induce its flowering prior to apply thereto a product or composition further inducing its flowering. The increase of the endogenous level would then have to be compared with the endogenous level of the genetically modified plant in which flowering has previously been induced.

According to a preferred embodiment of the invention, the endogenous level of a selected jasmonate compound is increased by:

a) applying to the plant at least one selected jasmonate compound and/or salts thereof;
b) applying to the plant at least one inhibitor of a sulfotransferase sulfonating 12-hydroxyjasmonic acid and/or 11-hydroxyjasmonic; and/or
c) applying to the plant at least one stimulator of an hydroxylase hydroxylating jasmonic acid and/or methyljasmonic acid.

More preferably, the selected jasmonate compound which is applied is 12-hydroxyjasmonic acid or 11-hydroxyjasmonic acid. However, amino acid

conjugates, glucosides, sulfate esters, salts, derivatives or any others natural or chemically synthesized compounds having a similar biological activity on flowering induction, is suitable according to the invention. For instance, inactive compounds such sulfate ester of 12-hydroxyjasmonic acid, sulfate ester of 12-hydroxymethyljasmonic acid, sulfate ester of 11-hydroxyjasmonic acid, and sulfate ester of 11-hydroxymethyljasmonic acid could be applied to a plant and it is possible that the bacterial or fungal flora of the plant or of its soil would hydrolyze these compounds in active jasmonate compounds. Any of the above mentioned compounds can be applied in a pure form or as a mixture of a plurality of compounds.

Inhibitors of hydroxyjasmonic acid sulfotransferase(s) should prevent *in vivo* inactivation of the flower-inducing molecule by sulfonation. To the contrary, stimulators of jasmonic acid hydroxylase(s) should help in the production of jasmonate family compound(s).

The above mentioned jasmonate compounds, stimulators and/or inhibitors can be part of a composition for inducing flowering in a plant. Such a composition would comprise a flowering inducing effective amount of at least one selected jasmonate compound, in combination with a diluent or a carrier. The compound(s) and their amount would be selected such that an early flowering of the plant would occur following application of the flower inducing composition when compared to a corresponding plant in the absence of said compound(s).

The carrier or diluent can be a solvent such as water, oil or alcohol. The composition may also comprise others active agents such as fertilizers and growth regulators. The inducing composition may also be formulated with emulsifying agents in the presence or absence of fungicides or insecticides, if required. The precise amount of compound employed in the practice of the present invention will depend upon the type of response desired, the formulation used and the type of plant treated. In the following examples, the plant culture medium was supplemented with about 10 µM of 12-hydroxyjasmonate or with 50 µM methyljasmonic acid for flowering induction.

i) Flowering retardation

According to the invention, flowering is delayed by lowering the endogenous level in a plant of at least one given compound of the jasmonate family selected preferably from the group consisting of jasmonic acid, jasmonic acid-tyrosine conjugate, jasmonic acid-tryptophan conjugate, jasmonic acid-phenylalanine conjugate, jasmonic acid-isoleucine conjugate, jasmonic acid-leucine conjugate, jasmonic acid-valine conjugate, 12-hydroxyjasmonic acid, glucoside of 12-hydroxyjasmonic acid, methyljasmonic acid, 12-hydroxymethyljasmonic acid, glucoside of 12-hydroxymethyljasmonic acid, 11-hydroxyjasmonic acid, glucoside of 11-hydroxyjasmonic acid, 11-hydroxymethyljasmonic acid, and glucoside of 11-hydroxymethyljasmonic acid. More preferably, the levels of 12-hydroxymethyljasmonic acid and of 11-hydroxymethyljasmonic acid are reduced. The flowering delay and the endogenous level lowering is detectable when compared to a corresponding plant in which the endogenous level of the compound has not been modified. However, it could be preferable in some cases to genetically modify a plant to delay its flowering prior to apply thereto a product or composition further delaying its flowering. The lowering of the endogenous level would then have to be compared with the endogenous level of the genetically modified plant in which flowering has previously been delayed.

According to a preferred embodiment of the invention, the endogenous level of a selected jasmonate compound is lowered by:

a) applying to the plant at least one inhibitor and/or an inactivator of a selected jasmonate compound;
b) applying to the plant at least one stimulator of a sulfotransferase sulfonating 12-hydroxyjasmonic acid and/or 11-hydroxyjasmonic acid; and/or
c) applying to the plant at least one inhibitor of an hydroxylase hydroxylating jasmonic acid and/or methyljasmonic acid.

Inhibitor(s) and/or an inactivator(s) of jasmonate compounds should block or inhibit the biological activity of jasmonate compound(s).

Stimulator(s) of hydroxyjasmonic acid sulfotransferase(s) should stimulate *in vivo* inactivation of the flower-inducing molecule by sulfonation. To the contrary,

inhibitors of jasmonic acid hydroxylase(s) should prevent the production of hydroxylated jasmonate compound(s).

As for the flowering compounds, the above stimulators and/or inhibitors can be applied in a pure form, as a mixture of a plurality of compounds or be part of a flowering delaying composition.

2) Molecular approach

In accordance with the present invention, genetic sequences encoding a plant hydroxyjasmonic acid sulfotransferase have been identified, cloned and used to generate transgenic plants.

SEQ ID NO 1 (Fig. 7; GenBank™: accession number AB010697, nucleotides 53936 to 55015; and Stanford University *Arabidopsis thaliana* database: clone number M0J9, gene MOJ9.16 and EST 119G6T7) corresponds to the gene *AtST2a* in *Arabidopsis thaliana*. SEQ ID NO 3 (Fig. 8) is an amino acid sequence deduced from SEQ ID NO 1. This amino acid sequence is of public domain and comes from the Kazusa Arabidopsis Opening Site (KAOS) of the Kazusa DNA Research Institute (KDRI) (http://www.kazusa.or.jp/kaos/; clone number M0J9, gene MOJ9.16). The present inventors have found that the *AtST2a* gene from *Arabidopsis thaliana* encodes a sulfotransferase that sulfonates 12-hydroxyjasmonic acid and 11-hydroxyjasmonic acid with high specificity. Although not shown, results obtained demonstrated that this hydroxyjasmonic acid sulfotransferase exhibits high affinity for its substrate with a Km value of 11 µM for 12-hydroxyjasmonic acid and 60 µM for 11-hydroxyjasmonic acid. The enzyme did not accept structurally related compounds such as cucurbic acid, arachidonyl alcohol or prostaglandins. Maximum enzyme activity was observed at pH 7.5 in Tris/HCl buffer and did not require divalent cations for activity. The purified recombinant protein expressed in *E. coli* migrated in SDS-PAGE at a position corresponding to approximately 35,000 daltons (see Fig. 4).

SEQ ID NO 2 (Fig. 9; GenBank™: accession number AB010697, nucleotides 50627 to 51670; and Stanford University *Arabidopsis thaliana* database: clone number M0J9 gene MOJ9.15), corresponds to the gene *AtST2b* in *Arabidopsis thaliana*. SEQ ID NO 4 (Fig. 10) is an amino acid sequence deduced from SEQ ID

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NO 1. This amino acid sequence is of public domain and comes from the Kazusa Arabidopsis Opening Site (KAOS) of the Kazusa DNA Research Institute (KDRI) (http://www.kazusa.or.jp/kaos/; clone number M0J9, gene MOJ9.15). Amino acid sequence alignment between SEQ ID NOS 3 and 4 indicates that they share 85% amino acid sequence identity and 92% similarity, suggesting that *AtST2a* and *AtST2b* are functional homologues encoding isoenzymes.

Accordingly, one aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a plant hydroxyjasmonic acid sulfotransferase enzyme. More particularly, the present invention is directed to an isolated nucleic acid molecule comprising a nucleotide sequence preferably selected from the group consisting of SEQ ID NO:1, nucleotide sequences having at least 50% similarity with SEQ ID NO:1, SEQ ID NO:2, nucleotide sequences having at least 50% similarity with SEQ ID NO:2, and sequences hybridizing under low stringency conditions to one or more of theses sequences.

The nucleic acid molecules contemplated herein may exist in either orientation alone or in combination with a vector and preferably an expression-vector capable of facilitating transfer and expression of the nucleic acid into the plant cell and/or facilitating integration into the plant genome. Such a vector may, for example, be adapted for use in electroporation, microprojectile bombardment, Agrobacterium-mediated transfer or insertion via DNA or RNA viruses. The vector and/or the nucleic acid molecule contained therein may or may not need to be stably integrated into the plant genome. The vector may also replicate and/or express in prokaryotic cells. Preferably, the vector molecules or parts thereof are capable of integration into the plant genome. The nucleic acid molecule and/or the vector may additionally contain a promoter sequence capable of directing expression of the nucleic acid molecule in a plant cell. The nucleic acid molecule and/or the vector may also be introduced into the cell by any number of means such as those described above. The vector may also comprise a genetic sequence encoding a ribozyme capable of cleaving a hydroxyjasmonic acid sulfotransferase mRNA transcript.

The present invention is exemplified using nucleic acid sequences derived from *Arabidopsis thaliana* since this plant is commonly studied in and it represents a convenient and easily accessible source of material. However, one skilled in the art will immediately appreciate that similar sequences can be isolated from any number of sources such as other plants or certain microorganisms (e.g. fungi or bacteria). All such nucleic acid sequences encoding directly or indirectly a hydroxyjasmonic acid sulfotransferase are encompassed by the present invention regardless of their source. Examples of other suitable sources of genes encoding hydroxyjasmonic acid sulfotransferase include, but are not limited *to Brassica napus, Brassica oleracea* and *Brassica juncea*.

i) *Flowering induction*

An aspect of the invention contemplates a plant genetically modified to flower early when compared to a corresponding plant not genetically modified, wherein the genetically modified plant has an increased endogenous level of at least one given compound of the jasmonate family selected preferably from the group consisting of jasmonic acid, jasmonic acid-tyrosine conjugate, jasmonic acid-tryptophan conjugate, jasmonic acid-phenylalanine conjugate, jasmonic acid-isoleucine conjugate, jasmonic acid-leucine conjugate, jasmonic acid-valine conjugate, 12-hydroxyjasmonic acid, glucoside of 12-hydroxyjasmonic acid, methyljasmonic acid, 12-hydroxymethyljasmonic acid, glucoside of 12-hydroxymethyljasmonic acid, 11-hydroxyjasmonic acid, glucoside of 11-hydroxyjasmonic acid, 11-hydroxymethyljasmonic acid, and glucoside of 11-hydroxymethyljasmonic acid, when compared to the corresponding non-genetically modified plant.

In a preferred embodiment, the endogenous level of the selected jasmonate compound is increased by:

a) increasing in the plant the endogenous level of an hydroxylase hydroxylating jasmonic acid and/or methyljasmonic acid; and/or
b) lowering in the plant the endogenous level of a sulfotransferase sulfonating 12-hydroxyjasmonic acid and/or 11-hydroxyjasmonic acid.

According to a preferred embodiment of the invention this is achieved by genetically modifying the plant so as to lower the expression of the sulfotransferase sulfonating 12-hydroxyjasmonic acid and/or 11-hydroxyjasmonic acid, and functional homologues of this sulfotransferase. More preferably, the plant is modified for inhibiting or blocking the expression of at least one gene selected from the group consisting of *AtST2a*, *AtST2b* and functional homologues of *AtST2a* or of *AtST2b*.

Many methods for inhibiting expression of genes in plants are well known in the art, such as techniques using ribozymes, targeted mutagenesis, T-DNA insertion mutagenesis, and antisense techniques to name a few, and these methods could be used to reduce the present invention in practice. According to a preferred embodiment of the invention, the expression of one of the above mentioned gene is inhibited by providing a transgenic plant expressing an exogenous nucleic acid sequence antisense to this gene. More preferably, the endogenous level of the sulfotransferase sulfonating 12-hydroxyjasmonic acid and/or 11-hydroxyjasmonic acid is lowered by expressing into a genetically modified plant an exogenous nucleic acid sequence, the exogenous nucleic acid sequence encoding i) for a nucleic acid sequence antisense to a gene encoding at least one of said sulfotransferases or ii) for a nucleic acid sequence antisense to a fragment of this gene.

Accordingly, another aspect of the invention contemplates a method for producing a transgenic plant with reduced endogenous or existing hydroxyjasmonic acid sulfotransferase activity, such transgenic plant thereby being capable of flowering early. Preferably, the altered level would be less than the endogenous or existing level of activity in a comparable non-transgenic plant.

According to one embodiment, the method comprises the steps of:

a) introducing into a cell of a suitable plant an exogenous nucleic acid molecule comprising a sequence of nucleotides antisense to a sequence encoding a plant hydroxyjasmonic acid sulfotransferase, preferably a 11- or 12-hydroxyjasmonic acid sulfotransferase;
b) regenerating a transgenic plant from the cell; and where necessary

c) growing the transgenic plant for a time and under conditions sufficient to permit expression of the antisense sequence and thereby inhibiting expression of the hydroxyjasmonic acid sulfotransferase.

In a related embodiment, the method for producing a transgenic plant with reduced endogenous or existing hydroxyjasmonic acid sulfotransferase activity comprises the step of altering the hydroxyjasmonic acid sulfotransferase gene(s), preferably the 11- or 12-hydroxyjasmonic acid sulfotransferase gene, through modification of the endogenous sequences via homologous recombination from an appropriately altered hydroxyjasmonic acid sulfotransferase gene or derivative or part thereof introduced into the plant cell, and regenerating a transgenic plant from the cell.

ii) *Flowering retardation*

An aspect of the invention contemplates a plant, genetically modified to flower tardily when compared to a corresponding plant not genetically modified, wherein the genetically modified plant has a lowered endogenous level of at least one given compound of the jasmonate family selected preferably from the group consisting jasmonic acid, jasmonic acid-tyrosine conjugate, jasmonic acid-tryptophan conjugate, jasmonic acid-phenylalanine conjugate, jasmonic acid-isoleucine conjugate, jasmonic acid-leucine conjugate, jasmonic acid-valine conjugate, 12-hydroxyjasmonic acid, glucoside of 12-hydroxyjasmonic acid, methyljasmonic acid, 12-hydroxymethyljasmonic acid, glucoside of 12-hydroxymethyljasmonic acid, 11-hydroxyjasmonic acid, glucoside of 11-hydroxyjasmonic acid, 11-hydroxymethyljasmonic acid, and glucoside of 11-hydroxymethyljasmonic acid, when compared to the corresponding non-genetically modified plant.

In a preferred embodiment, the endogenous level of the selected jasmonate compound is lowered by:

a) lowering in the plant the endogenous level of an hydroxylase hydroxylating jasmonic acid and/or methyljasmonic acid; and/or
b) increasing in the plant the endogenous level of a sulfotransferase sulfonating 12-hydroxyjasmonic acid and/or 11-hydroxyjasmonic acid.

According to a preferred embodiment of the invention this is achieved by genetically modifying the plant so as to increase the expression of the sulfotransferase sulfonating 12-hydroxyjasmonic acid and/or 11-hydroxyjasmonic acid, or functional homologues of this sulfotransferase. More preferably, the plant is modified to increase the expression of at least one gene selected from the group consisting of *AtST2a*, *AtST2b* and functional homologues of *AtST2a* or of *AtST2b*.

Methods for increasing expression of genes in plants are well known in the art, such as activation tagging, transgenesis under the control of a strong promoter, and these methods could be used to reduce the present invention in practice. According to a preferred embodiment of the invention, the expression of one of the above-mentioned genes is increased by expressing into the plant a gene expressing the sulfotransferase sulfonating 12-hydroxyjasmonic acid and/or 11-hydroxyjasmonic under the control of a constitutive or an inducible promoter.

Accordingly, another aspect of the invention contemplates a method for producing a transgenic plant with increased endogenous or existing hydroxyjasmonic acid sulfotransferase activity, such transgenic plant thereby being capable of flowering tardily. Preferably, the altered level would be higher than the endogenous or existing level of activity in a comparably non-transgenic plant.

According to a preferred embodiment, the method comprises the step of:

a) introducing into a cell of a suitable plant an exogenous nucleic acid molecule comprising a sequence of nucleotides encoding a plant hydroxyjasmonic acid sulfotransferase, preferably a 11- or 12-hydroxyjasmonic acid sulfotransferase;
b) regenerating a transgenic plant from the cell; and where necessary
c) growing the transgenic plant for a time and under conditions sufficient to permit expression of the nucleic acid sequence into a plant hydroxyjasmonic acid sulfotransferase, preferably a 11- or 12-hydroxyjasmonic acid sulfotransferase.

* * *

The details of the construction of transgenic plants are known to those skilled in the art of plant genetic engineering and do not differ in kind from those practices which have previously been demonstrated to be effective in tobacco,

petunia and other model plant species (e.g. electroporation, microprojectile bombardment, Agrobacterium-mediated transfer or insertion via DNA or RNA viruses). One skilled in the art will immediately recognize the variations applicable to the methods of the present invention, such as increasing or decreasing the expression of the sulfotransferase naturally present in a target plant leading to modulation of flowering to this plant. The present invention, therefore, extends to all transgenic plants containing all or part of the nucleic acid sequence of the present invention, or antisense forms thereof and/or any homologues or related forms thereof and in particular those transgenic plants which exhibit altered flowering properties.

The transgenic plants may contain an introduced nucleic acid molecule comprising a nucleotide sequence encoding or complementary to a sequence encoding a hydroxyjasmonic acid sulfotransferase. Generally, the nucleic acid would be stably introduced into the plant genome, although the present invention also extends to the introduction of a hydroxyjasmonic acid sulfotransferase nucleotide sequence within an autonomously-replicating nucleic acid sequence such as a DNA or RNA virus capable of replicating within the plant cell. The invention also extends to cut flowers and seeds from such transgenic plants.

* * *

A further aspect of the present invention is directed to an isolated plant hydroxyjasmonic acid sulfotransferase, and more particularly to an isolated hydroxyjasmonic acid sulfotransferase selected from the group of:

a) an enzyme whose amino acid sequence is represented by SEQ ID NO 3 or SEQ ID NO 4; and
b) functional homologues of enzyme a), isolated from a plant or derived from enzyme a) by substitution, deletion or addition of one or several amino acids in the amino acid sequences defined in a), and having similar biological activity or function(s).

These enzymes may be purified from plants or produced with routine recombinant techniques using SEQ ID NO 1, SEQ ID NO 2, or portion(s) thereof. Various methods of purification and molecular biology techniques for producing recombinant proteins are described in the art such that a skilled technician could

obtain these enzymes without large amounts of trial and error, or complicated experimentation. Isolated hydroxyjasmonic acid sulfotransferase will provide a source of material for research to develop, for example, more active enzymes and to produce antibodies binding with affinity thereto.

Accordingly, a related aspect of the invention is directed to antibodies binding with affinity to one or more of the above mentioned hydroxyjasmonic acid sulfotransferases. Persons skilled in the art are aware that antibodies can be made against virtually any protein and should be capable of producing such antibodies using conventional techniques. Antibodies binding to hydroxyjasmonic acid sulfotransferases could be particularly useful in flowering retardation compositions and also for studying the biological activity of this type of enzymes.

EXAMPLES

The following examples are illustrative of the wide range of applicability of the present invention. The invention is not restricted to the control of flowering in *Arabidopsis thaliana* but can be applied to various plant species. It should readily occur that the recognition of activation or retardation of flowering using the compositions, and methods according to the present invention in connection with other plants not specifically illustrated herein, is readily within the capabilities of one skilled in the art. The following examples are intended only to illustrate the invention and are not intended to limit its scope. Modifications and variations can be made therein without departing from the spirit and scope of the invention.

The following experimental procedures and materials were used for the examples set fort below.

A) Materials And Methods

<u>Growing of plants</u>

A. thaliana plants of ecotype Columbia (ColO) and C24 were used for this study. The plants were grown in soil in a growth chamber during a 16-hour photoperiod, at a day-time temperature of 24 °C and a night-time temperature of 20 °C. For some experiments, the plants were grown in magenta boxes under sterile conditions according to the following protocol. Seeds of *Arabidopsis*

thaliana were sterilized for 5 minutes in a solution containing 1.5% sodium hypochlorite and 0.02% SDS, and washed five times in sterile water. Seeds were vernalized for four days at 4 °C. Seeds were then spread on agar-solidified medium containing Murashige and Skoog salts, 1% sucrose and vitamins.

Studies using a vector:

For transgenic studies a EcoR1-HindIII cassette, from the plasmid pBI-525 comprising two CaMV 35S promoters in tandem followed by an AMV translational enhancer and a NOS terminator, was ligated to the plasmid pBI-101 which was previously digested with the same restriction endonucleases. The resulting vector called pBI-101-525 contained two CaMV 35S minimal promoters in tandem followed by an AMV translational enhancer, a NOS terminator and a kanamycin resistance gene. *AtST2a* cDNA (SEQ ID NO 1; Fig. 7) was cloned both in the sense and the antisense orientation at the BaMHI site in a polylinker lying downstream of the AMV enhancer. Various other promoters may be used to drive the expression of an exogenous gene in a plant. For example the ubiquitin promoter may be used for constitutive expression. Alternatively, inducible promoters may also be used such as the ethanol-inducible promoter or the glucocorticoid-inducible promoter.

Agrobacterium transformation:

A. tumefaciens strain GV3101 pMP90 was transformed with the *AtST2a*-pBI-101-525 sense and antisense constructs by the method described in Gynheung *et al.* (1988) Biology Manual, A3:1-19.

Arabidopsis transformation:

A. thaliana plants of ecotype Columbia (ColO) were transformed with Agrobacterium containing the *AtST2a* gene in the sense orientation by the vacuum infiltration method as described previously in Benchtold *et al.* (1993), CR Acad. Sci. Paris, Life Sciences, 316: 1194. *A. thaliana* plants of ecotype C24 were transformed with the pBI-101-525 vector containing the *AtST2a* gene in the antisense orientation by the root explant method as described in Valvekans *et al.*

(1988) Proc Natl Acad Sci USA, 85: 5536. Seeds were collected from the T_0 plants, their surface was sterilized and transformants were selected on MS salt medium containing vitamins and supplemented with 50 µg/ml of kanamycin. For phenotypic analysis of the transgenic plants, the T_2 or T_3 seeds were vernalized for four days at 4 °C. Seeds were then spread on agar-solidified medium containing Murashige and Skoog salts, 1% sucrose and vitamins. Alternatively, the vernalized seeds were planted in soil and grown in a growth chamber under a 16-hour photoperiod, at a day temperature of 24 °C and a night temperature of 20 °C.

Western blot of protein extracts

Protein extracts from wildtype and transgenic plants were subjected to 12% SDS-PAGE. Following electrophoresis, the proteins were transferred to nitrocellulose membranes using a Bio-Rad™ semidry transblot apparatus according to the manufacturer instructions. Blots were incubated with rabbit anti-*ATST2a* primary antibodies. Immunodetection was carried on with alkaline phosphatase-conjugated anti rabbits antibodies and the immunodetection kit from Bio-Rad™.

Quantification of endogenous levels of 12-hydroxyjasmonate and 12 hydroxyjasmonate sulfate from Arabidopsis plants

i) 12-hydroxyjasmonate

Fresh plant material (1g) was homogenized with 10 ml methanol and 100 ng 12-($^2H_3$)OAc-jasmonate as internal standard, the filtrate was evaporated and acetylated with Py/Ac_2O at 20 °C overnight. The evaporated mixture was loaded on a 3 ml DEAE-Sephadex™ A25 columns (Acetate-form, methanol) and the column washed with 3 ml of methanol. After washing with 3 ml of 0.1 M acetic acid in methanol, fractions eluted with 5 ml of 1 M acetic acid in methanol were collected, evaporated and separated on preparative HPLC and analyzed by GC-MS.

HPLC: Eurospher™ 100-C_{18}, (5 µm, 250 x 4 mm), elution with a mixture methanol - 0.2 % acetic acid in H_2O (1 : 1) at a flow rate of 1 ml min^{-1}, UV detector 210 nm, fractions between R_t 5-6.5 min were evaporated.

Derivatization: Samples were dissolved in 200 µl $CHCl_3$/*N,N*-diisopropylethylamine (1 : 1) and derivatized with 10 µl pentafluorobenzylbromide at 20 °C overnight. The evaporated samples were dissolved in 5 ml n-hexane and passed through a SiOH-column (500mg; Machery-Nagel™). The pentafluorobenzyl esters were eluted with 7 ml n-hexane / diethylether (2 : 1), evaporated, dissolved in 100 µl MeCN and analysed by GC-MS

GC-MS: (GCQ Finnigan™), 70 eV, NCI, ionization gas NH_3 , source temperature 140°, column Rtx-5 (30 m x 0.25 mm, 0.25 µm film thickness), injection temperature 250°C, interface temperature 275°; Helium 40 cm s^{-1}; splitless injection; column temperature program: 1 min 60°C, 25°min^{-1} to 180° C, 5° min^{-1} to 270° C ,1 min 270 °, 10° min^{-1} to 300°, 25 min 300° .

ii) 12-hydroxyjasmonic acid sulfate

The negative ion electrospray (ES) mass spectra were obtained from a Finnigan™ MAT TSQ 7000 instrument (electrospray voltage 4 kV; heated capillary temperature 220 °C; sheath gas: nitrogen) coupled with a Micro-Tech Ultra-Plus MicroLC™ system equipped with a RP18-column (4 µm, 1x100 mm, Ultrasep™). For the HPLC a gradient system was used starting from H_2O:CH_3CN 90:10 (each of them containing 0.2% HOAc) to 10:90 within 15 min followed by a 10 min isocratic period at a flow rate of 70 µl min^{-1}. The collision-induced dissociation (CID) mass spectra during the HPLC run were performed with a collision energy of 30 eV; collision gas: argon, collision pressure: 1.8 x 10^{-3} Torr. All mass spectra are averaged and background subtracted.

B) RESULTS

Example 1: Flowering induction by treating A. *thaliana* plants with 12-hydroxyjasmonic acid

Arabidopsis plants of ecotype Colombia (Col0) were grown in magenta boxes containing phytoagar and vitamins in a growth chamber under a sixteen hour photoperiod at a day-time temperature of 24 degrees and a night-time temperature of 20 degrees for a period of 18 days. The plants were then treated

with 10 μM of 12-hydroxyjasmonic acid (Fig. 2B) or with water as a negative control (Fig 2A) for a period of 6 days.

As seen in Figure 2B, plants treated with 12-hydroxyjasmonate flowered earlier (2 days) than the plants treated with water alone. Despite the fact that a treatment with 12-hydroxyjasmonic induces the hydroxyjasmonic acid sulfotransferase, early flowering is observed in the treated plants. The early flowering phenotype might be amplified if the treatment is coupled with an inhibitor of the hydroxyjasmonic acid sulfotransferase.

These results are of great economic importance since they show that it is possible to induce flower formation by the exogenous application of 12-hydroxyjasmonate and/or others compounds of the jasmonate family to crop plants. Therefore it shows that one may induce early flowering when required by a simple application of a selected flower inducer to plants, particularly 12-hydroxyjasmonate.

Example 2: Transgenic plants flowering tardily

In this example, *A. thaliana* plants genetically modified were created by inserting therein a nucleic acid molecule encoding the *AtST2a* gene in the sense orientation under the control of a constitutive promoter. The results demonstrate that a higher endogenous expression of the hydroxyjasmonic acid sulfotransferase encoded by this gene is effective to delay flowering.

Figure 3 shows the phenotype of wild type non transgenic Col0 *Arabidopsis* plants (WT) as compared to transgenic plants expressing the *AtST2a* gene under the control of the CaMV35S promoter 27 days after germination (S5, S6, S9, and S16). As shown in Figure 3, expression of *AtST2a* gene in transgenic *Arabidopsis thaliana* affects flowering time since all the transgenic lines exhibited delayed flowering as compared with non-transformed plants.

Fig. 4 shows a Western blot of protein extracts of these plants probed with anti-*AtST2a* antibodies. This figure clearly shows that the length of the delay is correlated with the level of expression of the transgene. This suggests that it is possible to vary the length of the delay by selecting transgenic lines expressing *AtST2a* at different levels. Delaying flowering time results in increased vegetative

growth and biomass which is a major advantage for crop such as lettuce, carrot, cabbage, sugar cane, sugar beet, to mention a few.

Table 1 hereinbelow also shows that a higher endogenous expression of the hydroxyjasmonic acid sulfotransferase results in higher endogenous level of 12-hydroxyjasmonate sulfate in the transgenic line S9.

TABLE 1:

	Wildtype (WT)	**Transgenic (S9)**
12-hydroxyjasmonate sulfate	211 peak area/g	2234 peak area/g

Example 3: Transgenic plants flowering early in non-inductive flowering conditions

In this example, *A. thaliana* plants genetically modified were created by inserting therein a nucleic acid molecule encoding the *AtST2a* gene in the antisense orientation under the control of a constitutive promoter. The results demonstrate that a lower endogenous expression of the hydroxyjasmonic acid sulfotransferase is effective to induce flowering.

Figure 5 shows the phenotype of wild type *Arabidopsis* plants of ecotype C24 (WT) as compared to transgenic plants expressing the *AtST2a* gene in the antisense orientation under the control of the CAMV35S promoter (TL 7-2-5). In this experiment, the plants were grown under short days which is non-inductive for flowering in *Arabidopsis thaliana.* Under these conditions the wildtype plants will flower after approximately 95 days of vegetative growth. The photograph was taken 65 days after germination and shows clearly an early flowering phenotype for the transgenic plants. As shown in this figure, inhibition of regular expression of the *AtST2a* gene in transgenic *Arabidopsis thaliana* affects flowering time since all the transgenic lines exhibited early flowering as compared with non-transformed plants.

Table 2 hereinbelow also shows that a lower endogenous expression of the hydroxyjasmonic acid sulfotransferase results in a higher endogenous level of 12-hydroxyjasmonate and in a lower endogenous level of 12-hydroxyjasmonate sulfate in plants.

TABLE 2:

	Wildtype (WT)	Transgenic (TL 7-2-5)
12-hydroxyjasmonate	7.7 ng/g	54.1 ng/g
12-hydroxyjasmonate sulfate	990 peak area/g	448 peak area/g

Interestingly, apart from early flowering, the growth behavior and the size of the transgenic plants could not be distinguished from the non-transformed control plants.

Example 4: Transgenic plants flowering early under favorable flowering conditions

Treatment with methyljasmonic acid of wild type Arabidopsis plants grown under favorable flowering day time conditions leads to elevated endogenous levels of both jasmonic acid and 12-hydroxyjasmonic acid (data not shown), conditions which should favor flowering. However, flowering was induced in an extent lower than what was anticipated (data not shown). As it will be explained hereinafter, a highly probable explanation for these results is that *AtST2a* gene expression is strongly induced under these favorable flowering day time conditions when treated with methyljasmonate thereby blocking the positive effects of the increase level of 12-hydroxyjasmonic acid.

To confirm this hypothesis, 15 days old *A. thaliana* plants and transgenic plants expressing the *AtST2a* gene in the antisense orientation under the control of a constitutive promoter were treated with 50 μM methyljasmonic acid for a period of nine days, and the plants were grown under favorable flowering day time conditions.

Figure 6 shows the effect of methyljasmonic acid treatment on the phenotype of wild type non transgenic C24 *Arabidopsis* plants (WT C24) as compared to transgenic plants expressing the *AtST2a* in the antisense orientation under the control of the CAMV35S promoter (TL 7-2-5), 24 days after germination. As shown in this figure, expression of *AtST2a* in the antisense orientation results

in lowered levels of the *AtST2* protein and allows the transgenic plants to flower early in presence of methyljasmonic acid.

This confirms that it is preferable, under certain conditions, to genetically modify a plant to induce its flowering prior to apply thereto a product further inducing its flowering.

Example 5: *AtST2a* gene expression is regulated by 12-hydroxyjasmonate

Fifteen days-old Arabidopsis plants (Col0) were grown in magenta boxes in presence or in absence of 12-hydroxyjasmonate for a period of 24 hours. At the end of the incubation period, the plants were frozen in liquid nitrogen, ground to a fine powder and total mRNAs were extracted using the kit from the company Qiagen™. The mRNA extracts were resolved by agarose gel electrophoresis, and transferred by capillarity to a nylon membrane. The blot was probed with the sequence encoding *AtST2a*.

The results presented in Figure 11 show that 12-hydroxyjasmonic acid induces the expression of the *AtST2a* gene and that the level of expression is proportional to the amount of inducer. Furthermore, the results show that the level of expression is very low in untreated plants. The induction of *AtST2a* expression by its substrate suggests that the level of 12-hydroxyjasmonic acid present in the plant is tightly controlled. This result is not surprising considering the important role of 12-hydroxyjasmonic acid in the induction of flowering.

Example 6: Expression of *AtST2a* is under the control of photoperiod.

Fifteen days old Arabidopsis plants grown under long day conditions were transferred in the dark. At different time intervals, plants were collected, frozen in liquid nitrogen, ground to a fine powder and total mRNAs were extracted using a kit from the company Qiagen™. The mRNA extracts were resolved by agarose gel electrophoresis, and transferred by capillarity to a nylon membrane. The blot was probed with the sequence encoding *AtST2a*.

The results presented in Figure 12 show that expression of *AtST2a* increases with time when the plants are kept in the dark reaching significant levels after 8 hours of dark treatment. This result suggests that plants monitor

photoperiod by modulating the level of 11- and 12-hydroxyjasmonic acids. When the plants are grown under short day conditions, the increased level of expression of *AtST2a* leads to the sulfonation of 11- and 12-hydroxyjasmonic acids resulting in delayed flowering. When the plants are grown under long day conditions, *AtST2a* is not expressed and the levels of 11- and 12-hydroxyjasmonic acids increase resulting in an early flowering time.

C) CONCLUSION

As shown in the above examples, *AtST2a* and *AtST2b* gene expression is induced after the application of 12-hydroxyjasmonate with a maximum of six hours after the beginning of the treatment. This pattern of induction demonstrates that the level of 12-hydroxyjasmonic acid is tightly regulated *in vivo* suggesting that 12-hydroxyjasmonic acid plays an important role in the plant. *AtST2a* and *AtST2b* gene expression is also induced when the plants are grown in the dark. The kinetic of accumulation of *AtST2a* and *AtST2b* mRNA is slow with a maximum observed after 12 hours in the dark. Furthermore, there is a fast decrease in *AtST2a* and *AtST2b* mRNA levels when the plants are transferred back to light. Taken together, these results suggest that the biological function of *AtST2a* and *AtST2b* is to modulate the activity of 12-hydroxyjasmonic acid and 11-hydroxyjasmonic acid in relation to the photoperiod. The model presented in the section "General overview of the invention" integrates the different results obtained and tries to explain the role of the hydroxylated jasmonates and of the *AtST2a* and *AtST2b* genes in the control of flowering time. According to this model, 11- and 12-hydroxyjasmonic acids are synthesized slowly into the leaves from jasmonic acid or from early fatty acids precursors. The accumulation of these metabolites up to a threshold value induces flowering. When the plants are growing under short day time conditions, *AtST2a* and/or *AtST2b* will be expressed during the night and will inactivate 11- and 12-hydroxyjasmonic acids by sulfonation. This mechanism will retard flowering time until the photoperiod is favorable. When the plants are growing under long day time conditions, the level of expression of *AtST2a* and/or *AtST2b* is low and 11- and 12-hydroxyjasmonic acids will accumulate to levels sufficient to induce flowering.

While several embodiments of the invention have been described, it will be understood that the present invention is capable of further modifications, and this application is intended to cover any variations, uses, or adaptations of the invention, following in general the principles of the invention and including such departures from the present disclosure as to come within knowledge or customary practice in the art to which the invention pertains, and as may be applied to the essential features hereinbefore set forth and falling within the scope of the invention or the limits of the appended claims.